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Trace elements and activity of antioxidative enzymes in *Cistus ladanifer* L. growing on an abandoned mine area

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Abstract The Mediterranean shrub *Cistus ladanifer* grows naturally in São Domingos (Portugal), an abandoned copper mine. High levels of trace elements in plants can generate oxidative stress increasing the activity of antioxidant enzymes. The aim of this work was to evaluate and compare As, Cu, Pb and Zn concentrations and the activity of the soluble and cell wall ionically bounded forms of the enzymes catalase, peroxidase and superoxide dismutase in leaves of *C. ladanifer*, collected in spring and summer, growing on São Domingos mine and on a non-contaminated area (Pomarão). São Domingos soils showed high total concentrations of As (2.6 g kg^{-1}) and Pb (7.3 g kg^{-1}) however the available fraction represented less than 1.5% of the total. *C. ladanifer* population from mine showed tolerance to Pb and Zn, which attain in leaves concentrations considered toxic for plants. The enzymatic activity of catalase, peroxidise and superoxide dismutase varied with plant populations and seasons, although with no particular trend, being specific to each trace element and enzyme cell localization. Catalase activity was evenly distributed between the soluble and ionically bounded forms, whereas the ionically bounded form of peroxidase predominated relatively to total activity, and the opposite was observed for superoxide dismutase. Spring and summer leaves from the two areas presented enzymatic activities in both fractions except to peroxidase soluble activities in leaves collected in summer. *C. ladanifer* enzymatic activity seems to be related with the co-existence of different stress factors (trace elements concentration, temperature, UV radiation and drought). The survival and growth of this species on contaminated mining soils is due to the presence of effective antioxidant enzyme-based defence systems.

Keywords Antioxidative enzymes · Adaptative capacity · *Cistus ladanifer* L. · Sulphide abandoned mine · Trace elements

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Introduction

Mining activity generates a large quantity of waste rocks and tailings, which are accumulated in the soil surface. These residues can release high amounts of trace elements. These chemical elements, when present in the soil solution or weakly adsorbed (exchangeable fraction) on soil solid phases, can be absorbed by plants and, consequently, accumulated in their tissues. High contents of trace elements induce symptoms of toxicity in most of the plants. However, many wild plant species found in mine spoils are adapted to the local conditions and are relatively tolerant to high concentrations of trace metals in soils.

Many environmental stresses, like those produced by trace elements, can cause directly or indirectly oxidative stress in plants, due to an increase in the production of reactive oxygen species (Panda and Choudhury 2005). Oxidative stress can induce lipid peroxidation, the activity of certain enzymes and cause membrane damage and cellular toxicity (Singh et al. 2004). When under oxidative stress, plants can produce or stimulate antioxidative enzymes, like catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), and non-enzymatic constituents that remove and neutralize the reactive oxygen species (Shah et al. 2001). SOD function is to catalyse the transformation of reactive oxygen species, generating H_2O_2 (Cao et al. 2004). CAT and POD are involved in the H_2O_2 removal, generated by SOD on plant stress situations (Grant and Loake 2000). Therefore, it is important to study the effect of trace elements in the activity level of these enzymes, as part of the plant adaptation and/or detoxification process.

Enzymes can be present in two main active forms within the plant cell in what concerns their cellular localization: soluble in the cell's aqueous media (soluble forms) or linked to cell wall components through electrostatic interactions (ionically bounded forms). The differentiation of these two forms is based on the treatment necessary for their extraction (McDougall and Morrison 1995). Soluble forms can be extracted with a low ionic strength buffer, while ionically bounded forms are only extracted with a high ionic strength extraction buffer (typically 1 M of NaCl or KCl) (Dunand et al. 2002). The different location and interaction of these two forms within the cell wall, can imply a different mobility and, possibly, different physiological roles. For instance, it has been hypothesized that the relative activity, substrate specificity, and isoform constitution of the two enzyme forms of POD, may be related to the developmental stage of plants and stress conditions, like attack by pathogens, wounding and chemical treatments (Penel and Greppin 1994). As far as we know, there is no information concerning the relative activity of the two enzymatic forms of CAT, POD and SOD in plants growing in the presence of high amounts of trace elements.

The aim of this study was to investigate the uptake of As, Cu, Pb, and Zn and the activity of the soluble and ionically bounded forms of the enzymes CAT, POD and SOD in leaves of *Cistus ladanifer* L. growing on a contaminated mine area from Portugal (São Domingos) in comparison to a non-contaminated area, in order to understand the role of trace elements in the plant enzymatic activity. Comparison of enzymatic activities was performed in leaves with different stages of development (young and mature leaves) and collected in different seasons (spring and summer).

Materials and methods

Site description

The study was carried out in two different locations: the mining area of São Domingos and the non-contaminated area of Pomarão (both in Alentejo, Portugal). São Domingos, located in the SE of Portugal, is an abandoned copper mine included in the Iberian Pyrite Belt (IPB). Exploitation has been known since the pre-roman times, and massive sulphides were exploited for Cu, Zn and S from 1868 until the exhaustion in 1966 (Custódio 1996). The large-scale mining combined with a continuous mining history produced large quantities of mining waste with a high potential of trace elements contamination. Pomarão is located in the SE of Portugal, about 18 km south of São Domingos mine.

The studied areas have a typical Mediterranean climate. In São Domingos and Pomarão, the annual average air temperature is 17.6°C and annual precipitation is 559 mm, being the wet period from November to March and the dry period from May to September.

Field sampling

Soil and plant samples were collected in the two studied areas: São Domingos and Pomarão. Soils in São Domingos are thin and were developed on mining wastes, composed mainly by gossaneous materials and host rocks, while in Pomarão, the soils were developed on schists and greywackes. Three composite soil samples, collected in April of 2005, were a homogenate of nine trial points, with 20 cm depth, from three adjacent plots (150 m^2 each) *per* studied area. Three composite samples of each leaves type (young and mature leaves) were collected from 15 *C. ladanifer* plants *per* plot in April (spring) and June (summer) of 2005.

Chemical analyses

Soil samples were homogenized manually, air-dried and sieved at 2 mm. The soil fraction (<2 mm) was characterized for (Table 1): pH in water suspension (1:2.5 w/v); organic carbon by wet combustion; cation exchange capacity (CEC) and exchangeable cations by 1 M ammonium acetate at pH 7; P and K extractable by Egner–Riehm method and total N by Kjeldahl method (Póvoas and Barral 1992).

Total contents of As, Cu, Pb and Zn (Table 1) were analyzed by atomic emission spectrometry with induced plasma (ICP-EAS) and instrumental neutron activation analysis (INAA) after acid digestion, $HClO_4 + HNO_3 + HCl + HF$, (Activation Laboratories 2006). The available fraction of the same elements in each soil sample

Table 1 Characteristic of soils from São Domingos mine (contaminated area) and the non-contaminated area of Pomarão

	São Domingos	Pomarão
pH (H_2O)	4.53 ± 0.19[b]	5.99 ± 0.06[a]
Organic C (g kg^{-1})	16.87 ± 4.62[a]	11.67 ± 2.90[a]
Total N (mg kg^{-1})	49.47 ± 8.35[a]	58.80 ± 3.05[a]
Extractable P (mg kg^{-1})	2.13 ± 0.73[a]	1.54 ± 0.17[a]
Extractable K (mg kg^{-1})	105.8 ± 20.52[a]	81.54 ± 1.60[a]
Exchangeable cations ($cmol_c$ kg^{-1})		
Ca	1.80 ± 0.37[a]	2.06 ± 0.29[a]
Mg	0.87 ± 0.15[b]	1.93 ± 0.10[a]
K	0.26 ± 0.05[a]	0.18 ± 0.005[a]
Na	0.07 ± 0.01[a]	0.07 ± 0.02[a]
CEC ($cmol_c$ kg^{-1})	9.31 ± 1.24[a]	7.78 ± 0.39[a]
Elements concentrations (mg kg^{-1})		
As total	2643 ± 610[a]	15.93 ± 0.40[b]
As available	0.24 ± 0.20[a]	0.01 ± 0.01[a]
Cu total	226 ± 14[a]	124.67 ± 40.20[b]
Cu available	11.26 ± 1.90[a]	5.88 ± 0.68[b]
Pb total	7343 ± 1972[a]	44.67 ± 9.61[b]
Pb available	114.34 ± 45.26[a]	0.90 ± 0.12[b]
Zn total	43.33 ± 11.85[a]	74.67 ± 16.07[a]
Zn available	<dl	<dl

Data (mean ± SD) from the same row followed by a distinct letter are significantly different ($P < 0.05$)

dl detection limit; *CEC* cation exchange capacity

(Table 1) was extracted with 0.01 M diethylene triamine pentacetic acid—DTPA (Lindsay and Norvell 1978).

In laboratory, leaves were carefully washed with abundant distilled water. Part of the samples were frozen in liquid nitrogen and stored until the enzymatic analyses. For elemental analysis, the remaining leaves were dried at 70°C, grinded and digested with concentrated nitric acid (69%) under pressure, during 10 h at 150°C.

Extractable soil solutions and plant extracts were analysed by atomic absorption spectrophotometry (Perkin–Elmer Model A Analyst 100, equipped with a deuterium lamp background correction system and an HGA-800 electrothermal atomizer) with graphite furnace (Cu and Pb), flame (Cu, Pb and Zn) or hydrates generation (As). All the analysis, except total trace elements in soils, were performed in triplicate.

Enzyme extraction and activity quantification

To obtain the soluble and cell wall ionically bounded enzyme fractions a sequential extraction was performed in triplicate based on Pang et al. (2003) and Ingham et al. (1998). For the enzymes extraction, 10 mL of a 50 mM phosphate buffer solution (pH 7.2) containing 1 mM of EDTA and 1% (w/v) of polyvinylpolypyrrolidone (PVPP), were added to 0.5 g of lyophilized and finely grounded leaves, with the extraction occurring during 15 min at 4°C under continuous stirring. The homogenate was centrifuged for 10 min at 22,000*g* at 4°C. The supernatant volume, which contains the soluble forms of the enzymes, was measured, frozen with liquid nitrogen, and stored until the quantification of CAT, POD and SOD activities.

To the pellet obtained from the extraction of the soluble fraction of the enzymes, were added 10 mL of a 50 mM phosphate buffer solution (pH 7.2), containing 1 mM of EDTA, 1% (w/v) of PVPP and 1 M of NaCl. Extraction occurred during 15 min at 4°C, with continuous agitation followed by the mixture centrifugation for 10 min at 22,000*g* and 4°C. This supernatant containing the cell wall ionically bounded forms of the enzymes was frozen and stored until further analysis. Substrate saturation and linear relation between enzyme activity and enzyme concentration was determined using commercial CAT, POD and SOD.

CAT (EC 1.11.1.6) activity was quantified based on the procedure described by Wong and Whitaker (2003) and Chance and Maehly (1955). Solutions of 0.1 M phosphate buffer (pH 7.0), 200 mM of H_2O_2 and water Milli-Q were previously incubated at 25°C. In quartz cuvettes were added 2 mL of phosphate buffer, 50–150 µL of enzymatic extract, 150 µL of H_2O_2 and water to complete a total volume of 3 mL. Reaction was started by the addition of H_2O_2. The decomposition of H_2O_2 was followed at 240 nm for 2 min and the slope of the linear portion of the curve relating absorbance at 240 nm with time was calculated (ΔAbs_{240} min^{-1}). The calculated slope was used to determine the activity of CAT (µmol H_2O_2 min^{-1} g^{-1} plant fresh weight) using the extinction coefficient (ε) for H_2O_2 of 36.0 M^{-1} cm^{-1} (Verma and Dubey 2003).

POD (EC 1.11.1.7) activity was quantified using guaiacol as substrate as described by Yuan and Jiang (2003) and Chance and Maehly (1955). Solutions of 135 mM of guaiacol, 0.1 M of phosphate buffer (pH 7.0), 200 mM of H_2O_2 and water Milli-Q were previously incubated at 25°C. In quartz cuvettes were added 1.2 mL of phosphate buffer, 500–1,000 µL of enzymatic extract, 30 µL of H_2O_2, 200 µL of guaiacol and water to complete a total volume of 3 mL. The reaction was started by the addition of guaiacol. The increase in absorbance was recorded at 420 nm for 5 min and the slope of the linear portion of the curve relating absorbance at 420 nm with time was calculated (ΔAbs_{420} min^{-1}). The calculated slope was used to determine the activity of POD (µmol of H_2O_2 consumed min^{-1} g^{-1} plant fresh weight) using the extinction coefficient (ε) of 2.60 mM^{-1} cm^{-1} for the oxidation product of guaiacol, tetraguaiacol (Verma and Dubey 2003).

SOD (EC 1.15.1.1) activity was assayed according to Sun and Zigman (1977) and Khopde et al. (2001). Solutions of 0.1 M of sodium carbonate-hidrogenocarbonate buffer (pH 10.0), 5 mM of epinephrine (pH 2.0) and water Milli-Q were incubated at 25°C. In quartz cuvettes were added 1.5 mL of buffer, 25–100 μL of sample, 300 μL of epinephrine and water Milli-Q to complete a total volume of 3 mL. The enzymatic reaction was started by the addition of epinephrine. The increase in absorbance was recorded at 320 nm for 1 min and the rate of epinephrine oxidation calculated by the slope (ΔAbs_{320} min^{-1}) of the linear portion of the curve relating absorbance at 320 nm with time. The rate of epinephrine auto-oxidation was calculated in the same conditions but without enzymatic extract. One unit of SOD was defined as the amount of enzyme required to reduce the epinephrine auto-oxidation rate by 50%.

Data analysis

The comparison of biochemical parameters between the two areas was analysed by a one way ANOVA and Tukey test ($P < 0.05$) using the statistical programme SPSS v13.0 (SPSS Inc., Chicago, IL, USA). Bivariate correlations of Pearson were used to relate the influence of trace elements on the enzymatic activities of the leaves.

Results

Soils characterization

Soils presented clay loam (São Domingos) and loam (Pomarão) texture, and pH in São Domingos soils was significantly lower (pH = 4.53) than in Pomarão soils (5.99). Soil organic carbon, total N and extractable P and K were similar for the two studied areas and their low concentrations indicate the low fertility of these soils. CEC did not show significant differences in soils from contaminated and non-contaminated areas and Ca and Mg dominated the cation exchange complex (Table 1).

São Domingos soils presented higher total contents of As, Cu and Pb than Pomarão, but Zn concentrations were similar between the two areas (Table 1). The available fraction of Cu and Pb in São Domingos soils, extracted with DTPA solution, was significantly higher than in the non-contaminated soils while the As available fraction was similar between the studied areas (Table 1). However, the concentrations of the soil available fractions were relatively low when compared with the corresponding total concentration in the soil (As: 0.01% of total; Cu: 5.0% of total; Pb: 1.5% of total). In both areas, the Zn available fraction was under the detection limit of the apparatus.

Trace elements in *Cistus ladanifer* leaves

Trace element concentrations were analyzed in young and mature leaves, collected in spring and summer (Table 2). In summer, mature and young leaves from both sites and mature spring leaves from São Domingos showed similar As concentrations, while in spring, mature leaves (ML) from Pomarão and young leaves (YL) from both areas showed the lowest As concentrations. Concerning Cu concentrations in leaves, no statistical differences ($P < 0.05$) were observed between leaves type, studied areas and seasons. The highest Pb concentrations were observed in summer ML from Pomarão while the lowest Pb concentrations were observed in spring YL from São Domingos. Comparing both areas, plants from São Domingos showed the highest Zn concentrations. For this contaminated area, ML always presented higher Zn content than YL and significant differences were observed between seasons. By contrast, in Pomarão area, no statistical differences ($P < 0.05$) occurred between YL/ML and seasons.

Enzymatic activities

The activities of soluble and ionically bounded CAT, POD and SOD were analyzed in young and mature leaves, collected in spring and summer (Fig. 1). CAT activity, both soluble and ionically bounded forms, was quantifiable in YL and ML collected in the two areas and seasons. The highest value of CAT activity was observed in spring ML and summer YL both from São Domingos. In the same area, CAT activities of both fractions were higher in ML than in YL collected in spring while the opposite occurred in summer. No significant differences were observed between CAT activities of Pomarão YL and ML, except between ionically bounded fraction in spring. In both areas and seasons, soluble CAT activity was higher than the ionically bounded activity (54–69% of the total activity), except for São Domingos ML collected in spring where the soluble fraction represented approximately 47% of the total activity.

In spring, POD activity was detected on both fractions from all leaves type, while in summer YL from both locations had no quantifiable soluble POD activity. In general POD activity was similar between YL and ML from both areas and seasons. In summer ML from both areas presented higher soluble POD activity than YL. POD activity was always higher in the ionically bounded fraction (from 54 to 100% of total activity).

SOD activity was detected on both soluble and ionically bounded fractions for all leaves type and seasons. In general, Pomarão leaves showed higher values of soluble SOD activity than São Domingos leaves. For both seasons, no statistical differences ($P < 0.05$) were observed in SOD

Table 2 Concentrations of As, Cu, Pb and Zn in *Cistus ladanifer* leaves from São Domingos mine (contaminated area) and the non-contaminated area of Pomarão

	Elements concentration (mg kg^{-1} DW)			
	As	Cu	Pb	Zn
Spring				
Young leaves				
São Domingos	0.64 ± 0.25^{b}	7.27 ± 0.31^{a}	34.57 ± 1.82^{c}	113.71 ± 23.22^{c}
Pomarão	0.18 ± 0.11^{c}	10.44 ± 4.42^{a}	41.23 ± 6.45^{bc}	67.80 ± 13.13^{d}
Mature leaves				
São Domingos	1.91 ± 0.69^{a}	9.57 ± 3.55^{a}	56.00 ± 7.10^{b}	156.46 ± 18.02^{ab}
Pomarão	0.86 ± 0.24^{b}	7.67 ± 2.33^{a}	51.13 ± 3.71^{b}	93.37 ± 8.04^{cd}
Summer				
Young leaves				
São Domingos	1.67 ± 0.78^{a}	8.88 ± 2.04^{a}	60.15 ± 7.01^{ab}	140.22 ± 13.30^{b}
Pomarão	1.75 ± 0.19^{a}	8.87 ± 1.57^{a}	72.10 ± 7.49^{a}	70.19 ± 5.98^{d}
Mature leaves				
São Domingos	2.47 ± 0.41^{a}	7.20 ± 1.44^{a}	72.44 ± 5.10^{a}	181.12 ± 17.00^{a}
Pomarão	1.79 ± 0.48^{a}	9.11 ± 1.52^{a}	55.01 ± 5.36^{b}	92.09 ± 6.00^{cd}

Data (mean ± SD) from the same column followed by a distinct letter are significantly different ($P < 0.05$)

DW dry weight

soluble activities between YL and ML from the same area. Similar SOD activities from ionically bounded fraction were observed in both areas and seasons. SOD activity was present mainly in the soluble fraction (more than 63% of the total activity).

Discussion

Soils properties and chemical composition depended on materials from which soils were developed: *gossan* in São Domingos and schists and greywackes in Pomarão. As expected, total contents of As, Cu and Pb in the soils from São Domingos were higher than those in the Pomarão soils and, according to Kabata Pendias and Pendias (2001), are considered toxic for the majority of plants. Nevertheless, the soil available fraction for the same elements was relatively low when compared with the total soil content and only Cu and Pb concentrations from São Domingos soils were higher than Pomarão soils.

Although the total contents of As, Cu and Pb in soils were higher in São Domingos than in Pomarão, this was not directly reflected in the concentrations of these trace elements in *C. ladanifer* leaves. This can be due to the quite low concentration of the trace elements in the soil available fraction, or a consequence of the combination of this fact and the possible accumulation of these chemical elements in *C. ladanifer* roots (not analysed) which reduces its translocation to leaves and/or the existence of a plant exclusion mechanism.

Arsenic concentration in YL and ML from both areas and seasons were below the concentration considered toxic for plants (5–20 mg kg^{-1}, Kabata Pendias and Pendias 2001). In both areas, spring ML showed higher As concentration than YL, as was also observed by Porter and Peterson (1975) for *Agrostis capillaris* L. and Tu and Ma (2002) for *Pteris vittata* L. growing on highly As contaminated soils. Apparently, in spring As is accumulated in mature leaves, protecting younger leaves from the As toxic effects. It can also reveal that As has low mobility in the phloem, thus limiting the As recirculation. Summer leaves from both areas showed higher As concentrations than spring leaves (except to São Domingos ML). This can be related with distinct As translocation rates into plant between seasons and also to a seasonal adaptation mechanism of the plant. *C. ladanifer* leaves collected in several mine areas from IPB also presented As concentrations in the same range (2.1–3 mg As kg^{-1}; Batista et al. 2004; Chopin and Alloway 2007; Freitas et al. 2004), besides the quite different As total concentration in the soils. The similar As concentration in *C. ladanifer* from different mine areas can indicate As tolerance mechanism of this plant species to high and different As soil content.

Although the Cu concentration (both total and available fraction) in soils from São Domingos was higher than in Pomarão, the Cu concentrations in *C. ladanifer* leaves were similar between the two sites, YL and ML and seasons (Table 2). The Cu concentration levels in leaves were comparable to the results obtained by Chopin and Alloway (2007) for the same plant species growing in mine soils of

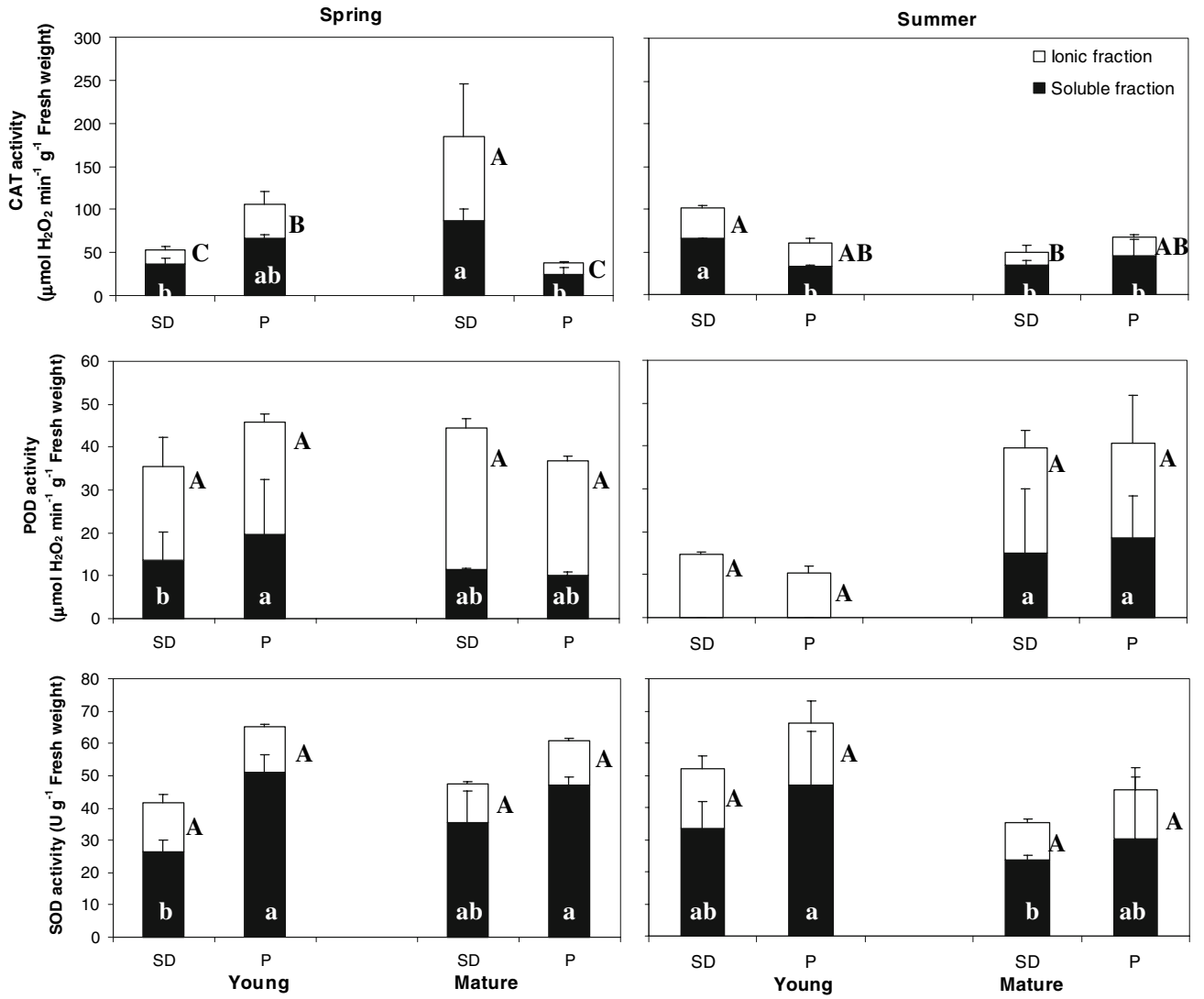

Fig. 1 Activities of soluble and cell wall ionically bounded catalase (*CAT*), peroxidase (*POD*) and superoxide dismutase (*SOD*) in *Cistus ladanifer* leaves collected in different seasons, growing on the abandoned mine of São Domingos (*SD*) (contaminated area) and the non-contaminated area of Pomarão (*P*)

Tharsis and Rio Tinto and within the range considered normal for various plant species (5–30 mg kg^{-1}; Kabata Pendias and Pendias 2001). The lack of differences between YL and ML and between seasons can indicate that Cu has a high internal mobility in the xylem and phloem (Clemens et al. 2002; Yruela 2005).

Although Pb available fraction in soils was significantly higher in São Domingos, Pb concentrations in plants were generally similar between the areas. Also, in both sampling areas and seasons, Pb concentrations in *C. ladanifer* leaves reached levels considered toxic for many plant species (30–300 mg kg^{-1}; by Kabata Pendias and Pendias 2001), which can indicate a high tolerance of this plant to the element because, apparently, the plants did not show any toxicity signs. The obtained results can suggest a possible protection mechanism for Pb in plants from São Domingos, characterized by different absorption rates, retention in roots and transport into plants (Yang et al. 1993; Sharma and Dubey 2005), due to the highest soil Pb available fraction with no significant changes on the plant Pb contents. Moreover, Pb concentration in leaves showed a tendency to be higher during the summer which can constitute a seasonal adaptation and suggest low Pb mobility in the phloem.

Zn concentrations in São Domingos leaves, collected in both seasons, exceed the critical toxicity content of 100 mg kg^{-1} for many plant species (Adriano 2001; Kabata Pendias and Pendias 2001), while in Pomarão leaves were within the normal range, although Zn soil available fraction in the two areas was lower than the detection limit. Microorganisms and root exudates can have enhanced element bioavailability and consequently

the elements were absorbed by plants (McGrath et al. 2001; Adriano et al. 2004). Zn uptake by the plants can also be due to chemical speciation of this element as well as soil characteristics. In São Domingos, Zn levels increased from YL to ML, suggesting a protection mechanism in YL from Zn toxic effects.

The published data concerning enzymatic activities of plants subjected to trace elements treatments were mainly performed under laboratory conditions with very few studies carried out, so far knows, in natural conditions. Thus, the study of the antioxidative defence systems of plants growing on polymetalic contaminated soils under field conditions can provide important clues on the biochemical tolerance of plants to high levels of different trace elements. This is the case of the present study, using a well adapted plant shrub, from the Mediterranean area, to mining soils with very high contents of As (2.6 g kg^{-1}) and Pb (7.3 g kg^{-1}).

In spring, the significant increase of CAT activity in both soluble and ionically bounded fractions observed in ML collected in São Domingos (Fig. 1), was correlated with an increase of Zn ($r = 0.70$) and As ($r = 0.75$) leaves concentration, respectively. The increase of Zn concentrations in leaves can induce CAT activity in order to protect the plant from oxidative stress, as reported by Van Assche and Clijsters (1990). The stimulation of total CAT activity due to Zn increase in leaves was also observed in *Vetiveria zizanioides* L. growing on Pb/Zn tailings under greenhouse conditions (Pang et al. 2003). It has also been reported by Mylona et al. (1998) that the total CAT activity in *Zea mays* was stimulated by exposure to arsenate and arsenite. Gadd and White (1989) found that absorbed As by plants was linked to cell wall components, what can cause the increase of the ionically bounded CAT activity. In fact, most of the CAT activity in the *C. ladanifer* leaves was in ionically bounded form. In contrast, the decrease observed on CAT activity in Pomarão, mainly in ionically bounded form, can be associated with the Zn increase to values close to those considered toxic for plants ($r = -0.75$ for soluble CAT and $r = -0.78$ for ionically bounded CAT) and to the lowest tolerance of *C. ladanifer* plants growing in the non-contaminated area. The CAT inhibition due to Zn toxicity was also observed by Panda (2003) in the moss *Taxithelium nepalense* growing on a growth chamber, which also presented low tolerance to Zn. This can be due to the inactivation of the enzyme protein function, changes in the assembly of enzyme subunits, decrease of total protein content due to inhibition of enzyme synthesis or combination of all these factors (MacRae and Fergunson 1985; Hertwig et al. 1992).

In summer, the soluble and ionically bounded CAT activities in both areas were generally lower and did not present strong correlation with trace elements contents in leaves. The reduction of CAT activities during the summer can be related to other factors like drought stress and increase of UV-B radiations. Alexieva et al. (2001) observed an inhibition of CAT total activity in *Pisum sativum* and *Triticum aestivum* plants when submitted to those stresses types.

POD activity was, in general, similar between YL and ML from both areas and seasons, except for YL in summer where soluble POD activity was below the detection limit, being the POD activity mainly linked to the cell wall. This can be due to the high summer temperatures, radiation level and drought stress in summer affecting young leaves from this Mediterranean region as was stated by several authors (Egert and Mitevini 2002; Larkindale et al. 2005). Although those environmental factors were found to affect POD activity, it was not reported if these effects affected the soluble or ionically bounded cell wall forms. Low values of POD activity in both areas in spring can be related with Zn concentrations in leaves which reached toxic concentrations ($r > -0.7$), as also observed by Panda (2003) and Choudhury and Panda (2004) for POD total activity. The ionically bounded POD activities from São Domingos plants in spring were related with the Zn concentration ($r = 0.86$), but in Pomarão leaves POD activities of this fraction was not correlated with any studied trace element. Also the variation of soluble and ionically bounded POD activity on leaves from the two areas, in summer, was not correlated with any quantified trace element. Most of the enzymatic activity was in the POD linked to cell wall (ionic fraction) where trace elements can be located in order to reduce their toxic effect in the cytoplasm.

SOD soluble fraction was the main contributor to total SOD activity in *C. ladanifer* for all leaves type, areas and seasons. SOD activity in YL and ML from both seasons was, generally, higher in Pomarão however these activities did not strong correlate with any studied trace element. SOD is important to catalyse reactive oxygen species, generating H_2O_2 which is then removed by the action of CAT and POD enzymes. *C. ladanifer* showed generally similar SOD activities (soluble and cell wall-bounded fraction) because, possibly, the oxidative stress was not so strong due to toxic concentration of Zn and Pb in leaves. In field conditions, the oxidative stress can be promoted by other stress factors like drought and high UV radiations and temperatures.

C. ladanifer showed high resistance to survive and grow in São Domingos mining area where soils can be considered extreme environments due to the elevated concentrations of As, Cu and Pb, low pH and fertility and, soil water deficiency in summer. Thus, the population from São Domingos would be more prone to oxidative stress. This resistance can be related with the adaptation and balance

between the generation of reactive oxygen species and their detoxification by CAT, POD and SOD, with possible different effect of the soluble and cell wall-bounded forms of these enzymes. This mechanism can represent in *C. ladanifer* plants a form of toxic elements tolerance. Different results for the studied elements contents in leaves and the enzymatic activities between YL and ML collected in spring and summer, suggest an adaptative response of *C. ladanifer* to the leaves developmental stage and environmental conditions (temperature, UV radiation and drought). The levels of activity of each form (soluble and ionically bounded) of CAT, POD and SOD enzymes varied with plant population, enzyme and season, and can also be related with the co-existence of different stress factors. CAT activity was evenly distributed between the soluble and ionically bounded forms. The main contribution for POD activity, was the ionically bounded form, while the opposite was observed for SOD. More studies are necessary, namely under greenhouse controlled conditions, to understand if *C. ladanifer* growing in São Domingos is a physiological ecotype specially adapted to high levels of trace elements.

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